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Preclinical evaluation of liposome-supported peritoneal dialysis for the treatment of hyperammonemic crises

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ABSTRACT

Liposome-supported peritoneal dialysis (LSPD) with transmembrane pH-gradient liposomes was previously shown to enhance ammonia removal in cirrhotic rats and holds promise for the treatment of hyperammonemic crises-associated disorders. The main objective of this work was to conduct the preclinical evaluation of LSPD in terms of pharmacokinetics, ammonia uptake, and toxicology to seek regulatory approval for a first-in-human study. The formulation containing citric acid-loaded liposomes was administered intraperitoneally at two different doses once daily for ten days to healthy minipigs. It was also tested in a domestic pig model of hyperammonemia. The pharmacokinetics of citric acid and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine was linear following intraperitoneal administration of medium and high dose. There was no systemic accumulation following daily doses over ten days. The systemic exposure to phospholipids remained low. Furthermore, the liposome-containing peritoneal fluid contained significantly higher ammonia levels than the liposome-free control, demonstrating efficient ammonia sequestration in the peritoneal space. This was indeed confirmed by the ability of LSPD to decrease plasmatic ammonia levels in artificially induced hyperammonemic pigs. LSPD was well tolerated, and no complement activation-related pseudoallergy reactions were observed. The safety profile, the linear pharmacokinetics of citric acid following repeated administrations of LSPD as well as the linear dose-dependent ammonia sequestration in the peritoneal space provide a strong basis for the clinical investigation of LSPD.

1. Introduction

Ammonia is a highly abundant metabolite implied in amino acid metabolism, protein degradation, and acid-base homeostasis [1]. As ammonia is neurotoxic, the urea cycle has evolved to control its systemic levels, acting as an efficient metabolic pathway in the liver [1]. Toxic increases in systemic ammonia levels mainly stem from inherited or acquired impairments in hepatic ammonia detoxification [1]. In hereditary hyperammonemia, one or several enzymatic defects due to inborn errors of metabolism hinder the detoxification capacity of the urea cycle (deficiencies in urea cycle enzymes and transporters, organic acidemias) [2,3], whereas acquired hyperammonemia is secondary to a liver injury (liver failure, hepatitis, drug-induced hepatotoxicity,

cirrhosis). Sustained elevated blood ammonia leads acute or chronic neurotoxicity resulting in adult patients in a severe neuropsychiatric disorder called hepatic encephalopathy (HE). Symptoms range from subtle cognitive impairment to altered mental status, hepatic coma and death [4]. Hyperammonemic crisis (HAC) associated to HE is a significant contributor of morbidity and often requires critical care management in the intensive care unit [4]. The mechanisms by which ammonia leads to irreversible cerebral damages involve dysregulated amino acid pathways and neurotransmitter systems, as well as cerebral energy metabolism, nitric oxide synthesis and oxidative stress [5].

As severe and prolonged HAC is associated with a poor prognosis, rapid detoxification measures aiming at lowering ammonia levels are crucial to reduce the risks of irreversible cognitive damage, long-term

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sequelae and death [6–8]. Ammonia scavengers such as sodium phenylbutyrate or the carbamoyl phosphate 1 stimulating agent carglumic acid are indicated for the long term treatment of inborn errors of metabolism affecting the urea cycle [2,9–11]. HAC are managed with hemofiltration or hemodialysis; however, due to the difficulty in establishing vascular access lines in neonates and the lack of appropriate equipment, pediatric patients must be transferred to highly specialized tertiary centers where this intervention is often initiated too late [12]. Current practice guidelines for the treatment of HE in adults with an underlying liver injury recommend the administration of the non-absorbable disaccharide lactulose, a laxative with pH-lowering and prebiotic effects, and the minimally absorbed antibiotic rifaximin, which target the microbial production of ammonia in the gut [4]. Lactulose and rifaximin are mostly effective for the maintenance therapy in chronic HE to reduce the recurrence of HAC but their clinical benefit for severe cases remains controversial [4]. Therefore, the development of novel treatments for early and effective management of HAC is warranted.

Recently, a novel approach for treating hyperammonemic patients was reported [13]. It was found that micrometer-sized transmembrane pH-gradient liposomes bearing an acidic core and administered *via* the peritoneal route sequestered ammonia in the peritoneal cavity (Fig. 1) and significantly attenuated brain edema in a rat model of secondary biliary cirrhosis [13,14]. The transmembrane pH-gradient allowed for a strong and efficient sequestration of ammonia while passively clearing other clinically relevant liver and uremic toxins [15,16]. Furthermore, the large size of the liposomes restricted their systemic bioavailability upon peritoneal administration [13,14,17]. The present study mainly aimed at characterizing in minipigs the toxicokinetics, ammonia uptake, and toxicology of two doses (medium and high) of peritoneally applied transmembrane pH-gradient liposomes, which were previously shown to display good efficacy in rats [13] in view of the first-in-human (FIH) clinical trial. This animal model was chosen due to its high sensitivity to colloids potentially leading to complement activation-related pseudoallergy (CARPA), which exceeds the reaction observed in humans [18]. A secondary objective of this study was to confirm ammonia capture in an artificially-induced hyperammonemic domestic pig model.

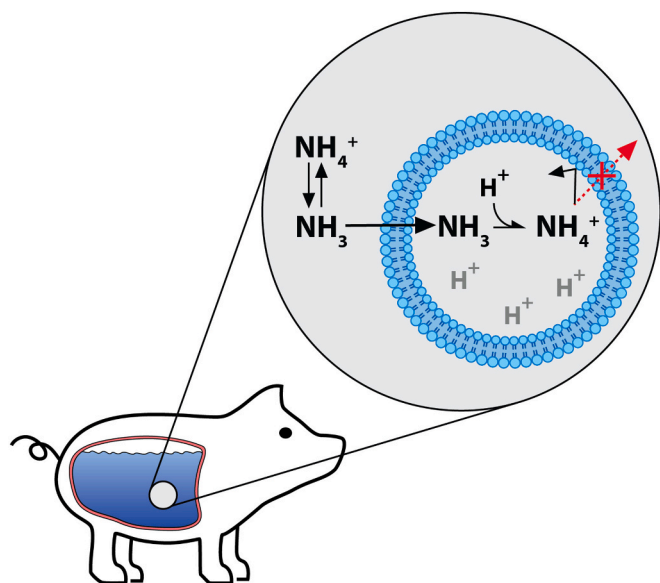


Fig. 1. Schematic depiction of peritoneal ammonia capture using transmembrane pH-gradient liposomes. The unprotonated form of ammonia readily diffuses across the liposomal bilayer. Upon entering the acidic liposomal lumen, it is protonated and subsequently trapped in the core due to its positive charge. Applied as a peritoneal dialysis solution, these liposomes can extract ammonia from the body.

2. Materials and methods

2.1. Materials

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)-2000] (mPEG-DSPE) were supplied by Corden Pharma Switzerland LLC (Liestal, Switzerland) or Lipoid GmbH (Ludwigshafen, Germany). Cholesterol (Chol) was purchased from Carbogen Amcis B.V. (Veenendaal, The Netherlands) or Sigma Aldrich (Buchs, Switzerland). Heparin was obtained from LEO Pharma (5000 IU/mL, Copenhagen, Denmark). Triton X100 and all other chemicals were purchased from Sigma Aldrich (St. Louis, MO). Physioneal® 35 Glucose was obtained from Baxter (Søborg, Denmark).

2.2. Methods and animal studies

2.2.1. Preparation of peritoneal dialysis fluids

For the repeated dose toxicity study, the liposome-supported peritoneal dialysis (LSPD) formulation was supplied as a 3-bottle kit each containing a citric acid solution, a liposomal aqueous suspension and a xylitol alkaline solution. The liposomal aqueous suspension was prepared according to a protocol which was developed for the scale-up production. Appropriate amount of water for injection was heated up to 60 °C in a jacketed stainless-steel vessel. A lyophilized lipid blend of DPPC:Chol:mPEG-DSPE (85.5:14:0.5 mol%) was then added into the vessel under mild stirring to reach a final lipid concentration of 100 mg/mL. After 2 h, the suspension was degassed prior to filling and was steam sterilized according to the Ph. Eur. guidelines (9th Edition, Chapter 5.1.1) (≥ 121 °C, ≥ 15 min). The citric acid (115 mg/mL, pH 2.0) and xylitol alkaline (21.3 mg/mL, pH 12.3) solutions were 0.2- μ m filtered and steam sterilized using similar conditions.

For the ammonia infusion study, the liposomal aqueous suspension was prepared by lipid film hydration method as previously described [13]. Briefly, the lipid mixture of DPPC, Chol, and mPEG-DSPE was dissolved at 85.5:14:0.5 mol% in $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (95:5, v/v). The lipid film was formed upon solvents evaporation in a rotary evaporator (KNF Neuberger, Freiburg, Germany) and kept under vacuum overnight. The film was then rehydrated with ultrapure water for 1 h at 56 °C, and the liposomal suspension (100 mM lipids) was degassed under vacuum, purged with argon, and sterilized by autoclaving for 20 min at 120 °C (Varioklav, H + P Labortechnik AG, Oberschleissheim, Germany). DPPC and Chol concentrations were quantified by high-performance liquid chromatography equipped with a charged aerosol detector (Dionex UltiMate® 3000, Thermo Scientific, Sunnyvale, CA) as previously described [13] and the amount of endotoxins by a commercial pyrogen test kit (Endosafe®-PTS™, Charles River Laboratories, Germany). The citric acid (pH 2, 1050 mOsm kg^{-1} , 600 mM) and xylitol alkaline (pH 12.4, 415 mOsm kg^{-1} , 135 mM) solutions were prepared as previously reported [15], vacuumed-filtered (0.22 μ m, TPP® Filtermax, Sigma Aldrich), autoclaved (20 min, 120 °C), and their endotoxin content quantified. The osmolality of each solution was verified (Osmomat 3000 basic freezing point osmometer, Gonotec GmbH, Berlin, Germany).

Immediately before use, the transmembrane pH gradient was generated *via* an osmotic shock as described previously [13,15]. Briefly, the citric acid solution was added to the liposome solution and gently agitated for at least 15 min. The acidic suspension was then added to the xylitol alkaline solution and gently mixed. The resulting suspension was left at room temperature for at least 10 min; the final pH (between 5 and 8) and visual aspect (milky) were controlled before use. The control solution Physioneal® 35 was reconstituted as per the manufacturer's instructions and infused within 24 h after mixing.

2.2.2. Animals

All the experiments conducted as part of the repeated dose toxicology study of LSPD were carried out in Göttingen minipigs. These

experiments were conducted in accordance with Executive Order No. 1245 of December 12th 2005 on Good Laboratory Practice (GLP) for Medicinal Products as required by the Danish Medicinal Agency and in compliance with OECD Principles of GLP. The study, which was carried out by Citoxlab Scantox A/S (Lille Skensved, Denmark), was performed in 23 minipigs (12 males and 11 females; Ellegaard Göttingen Minipigs A/S, Dalmose, Denmark) in a GLP facility. At arrival, the animals were approximately 3 to 4 months old and their body weight was between 6.7 and 9.5 kg for males and 7.4 to 10.7 kg for females. A pre-treatment period of at least 20 days was allowed including an acclimatization period of 5 days to the dosing set-up (e.g., jackets, tubing). The animals were fed in the morning prior to dosing and in the afternoon. They had *ad libitum* access to domestic quality drinking water.

The non-clinical proof of concept study in a large animal model was performed in domestic pigs (*Sus scrofa domestica*) at the University of Zurich (Tierspital, Vetsuisse Faculty, Zurich, Switzerland) in accordance with procedures and protocols approved by the cantonal veterinary authorities (protocol number of the license ZH159/15 26846) (see Section 2.2.8). The animals were acclimated for 7 days before the experiments, observed daily to ensure healthy status, and fasted overnight prior to anesthesia.

2.2.3. Surgical procedure and placement of catheter

At least one week before the repeated treatment initiation in Göttingen minipigs, catheters were aseptically implanted in the animals under general anesthesia with 40 mg azaperone/mL (0.05 mL/kg) and 1 mg atropine/mL (0.05 mL/kg) given as a single intramuscular injection, followed by an intramuscular injection in the neck of a mixture of Zoletil 50® Vet., Virbac, France (0.1 mL/kg body weight). In addition, the animals were given a subcutaneous injection of 5 mg meloxicam/mL (0.08 mL/kg) on the day of surgery and once daily for the following two days. Amoxicillin 150 mg/mL (0.1 mL/kg) was administered as an intramuscular injection on the day of operation and once daily for the following six days.

Incision into the peritoneal cavity was performed in the caudal flank of the animals. The coiled catheter (Covidien Peritoneal Prepritoneal Cuf Kt, Medtronic Denmark, Copenhagen, Denmark) was introduced through the incision and placed as far caudo-ventrally as possible to avoid being trapped by the omentum and to facilitate the evacuation of dialysate at the end of the peritoneal dialysis (PD) session. The catheter was tunneled subcutaneously and exited the skin dorso-laterally. The patency of the catheter was then tested by infusing and retrieving sterile warm saline solution. Finally, a heparin lock of 5 mL heparin (5 IU/mL) was infused. After closing the wound, the incision site was covered with bandage and a protective jacket was put on animals to minimize infections. For treatment of post-surgical pain, the animals were given a transdermal patch of fentanyl (25 µg/h) for three days.

2.2.4. Treatment regimen

Prior to commencement of repeated treatments, Göttingen minipigs were allocated to the LSPD group or the control group (Physioneal® 35 Glucose 1.36% w/v) according to Table 1. Dialysis sessions were performed daily for 10 days until the day before necropsy for the main study animals; a treatment free period of at least 14 days was added for the recovery animals. A volume of 30 mL/kg of the liposomal or control

PD fluid was infused over a period of 20 min through the implanted intraperitoneal catheter using peristaltic pumps. After a dwell time of 3 h \pm 20 min, the dialysate was recovered by gravity while the animals were restrained. Collection was continued until at least 75% of the test item had been retrieved or for a maximum of 10 min. Following collection, a heparin lock of 3 mL was infused into the catheter and the volume of dialysate recovered was measured.

2.2.5. Pharmacokinetics

2.2.5.1. Blood, LSPD and dialysate samples. On days 1 and 10, blood samples were taken from all Göttingen minipigs at the following timepoints: pre-treatment and 0.5, 1, 2, 3, 5, 7, 12, 16 and 24 h post-dose. On day 10, an additional blood sample was collected 48 h after start of treatment. Blood samples of approximately 3 mL were drawn from the jugular vein/bijugular trunk into vacutainers containing potassium ethylenediaminetetraacetate (EDTA) as anticoagulant. The vacutainer was placed in ice water until centrifugation (10 min, 1270 x g, 4 °C). Plasma samples were transferred to Nunc cryotubes (polypropylene tubes with internal thread, Thermo Scientific, Denmark) and then frozen at -70 °C or below within 1 h after collection pending analysis.

On the first (day 1) and last days of treatment (day 10), two samples of 3 mL were collected from the LSPD fluid (test item) before administration (time 0 h). In addition, two samples were collected from the dialysate in Nunc cryotubes at 1, 2 and 3 h \pm 5 min after the initiation of treatment and were immediately placed in ice water. The test item and dialysate samples were stored frozen at -70 °C or below within 1 h after collection pending analysis of citric acid, DPPC and cholesterol.

Citric acid, DPPC and cholesterol concentrations in plasma, in LSPD and in the dialysate were determined by Atlanbio (Saint Nazaire, France) in compliance with the GLP regulations. Validated bioanalytical method were based on protein precipitation followed by liquid chromatography with tandem mass spectrometry (LC-MS/MS). The sampling volume was of 10 µL. Each run included 8 non-zero calibration standards extracted in duplicate from the lower limit of quantitation (LLOQ) to the upper limit (UL) and two Quality Controls (QC) at each QC level. Calibration curve and QCs' nominal concentrations for each method are presented in the supporting information (SI) section. The LC-MS/MS analysis was carried out with a Shimadzu liquid chromatography system and autosampler (Shimadzu, Kyoto, Japan), an analytical chromatographic column (Phenomenex Kinetex EVO C18 2.6 µm 50*2.1 mm), and a triple quadrupole mass spectrometer system (API 3000, Sciex, Framingham, MA). Analyst software was used for LC-MS/MS instrument control, data acquisition and chromatogram peak integration. Watson® LIMS software (Thermo Electron, Philadelphia, PA) was used for sample and data management including regression, concentration calculations and statistics. All concentrations were generated from runs that fulfilled the predefined acceptance criteria and sample assay was completed within the time period for which stability data were available. Using LC-MS/MS, endogenous and administered citric acid, cholesterol, and DPPC cannot be discriminated. However, as the basal levels of these two components are generally stable, any increase following the administration of the liposomes can be related to the therapeutic intervention.

Table 1
Treatment groups in the Göttingen minipig study.

Group	Treatment identification	Citric acid/lipids (mg/mL)	Dose (mg/kg)	Number of animals
1	Control	–	0	10 (5 males, 5 females including 2 recovery animals) ^a
2	LSPD medium dose	Citric acid: 3.54 Lipids: 6.49	Citric acid: 106 Lipids: 195	7 (4 males, 3 females)
3	LSPD high dose	Citric acid: 4.66 Lipids: 10.6	Citric acid: 140 Lipids: 318	8 (4 males, 4 females including 2 recovery animals)

^a 2 animals (1 male and 1 female) did not complete the study due to catheter malfunction.

2.2.5.2. Pharmacokinetic calculations (citric acid, DPPC and cholesterol). Individual plasma and dialysate concentration *versus* time profiles of citric acid, DPPC and cholesterol were subjected to pharmacokinetic (PK) analysis using the software Phoenix WinNonlin Version 6.3 (Pharsight Corporation, Mountain View, CA). A non-compartmental analysis using WinNonlin model 200 (extravascular bolus dose model) was performed. When the plasma or dialysate concentrations were below the LLOQ, the data point was entered as the value of the LLOQ (i.e., 0.500 µg/mL for DPPC and 10.0 µg/mL for cholesterol and citric acid).

2.2.5.3. Plasma concentration profiles. The following PK parameters were determined from the plasma concentration profiles of citric acid, DPPC and cholesterol: the maximum plasma concentration (C_{max}) and the time when it occurred (t_{max}) were estimated by visual inspection of the data. The area under the plasma concentrations *vs.* time curve from time zero to 3 h, 24 h and time at last measurable concentration C_{last} post-dose (AUC_{0-3} , AUC_{0-24} and AUC_{0-t} , respectively) were calculated according to the linear/log trapezoidal method. The area under the curve from time zero to infinity (AUC_{inf}) was calculated with Eq. (1):

$$AUC_{inf} = AUC_{0-t} + C_{last}/\lambda_z \quad (1)$$

where λ_z is the elimination rate constant determined by linear regression using at least 3 data points from the terminal phase of the curve; an R^2 above 0.80 was deemed acceptable. The half-life $t_{1/2}$ was calculated with Eq. (2):

$$t_{1/2} = \ln 2/\lambda_z \quad (2)$$

The apparent systemic clearance (CL/F) and apparent volume of distribution (V_z/F) were calculated according to Eqs. (3) and (4), respectively:

$$CL/F = Dose/AUC_{inf} \quad (3)$$

$$V_z/F = Dose/(\lambda_z \times AUC_{inf}) \quad (4)$$

Furthermore, the accumulation ratio (R_A) following repeated administrations was calculated using Eq. (5):

$$R_A = AUC_{day10}/AUC_{day1} \quad (5)$$

using either AUC_{0-t} or AUC_{0-24} .

2.2.5.4. Dialysate concentration profiles. The PK parameters were determined from the dialysate concentration profiles of citric acid, DPPC and cholesterol analogously. The area under the dialysate concentration *vs.* time curve from time zero to 3 h post-dose (AUC_{0-3}) was calculated according to the linear/log trapezoidal method. Furthermore, the amount of each compound recovered at 3 h post-dose was calculated using the concentration at 3 h post-dose and the volume recovered at the end of the dialysis session; the corresponding %dose recovered at 3 h post-dose was calculated using Eq. (6):

$$\%Dose\ recovered = Amount\ recovered \times 100/Total\ dose\ administered \quad (6)$$

2.2.6. Efficacy

2.2.6.1. Blood and dialysate samples. On the first (day 1) and last days (day 10) of repeated treatments, blood samples of approximately 3 mL were drawn from the jugular vein/bijugular trunk of Göttingen minipigs. The samples were collected pre-dose as well as 1, 2, 3 (sampled before start of drainage) and 5 h after start of treatment into vacutainers containing K₃EDTA as anticoagulant. The vacutainer was placed in ice water until centrifugation (10 min, 1270 × g, 4 °C). Each plasma sample was then transferred to Nunc cryotubes and ammonia was analyzed within 2 h after sample collection.

At days 1 and 10, dialysate samples of 3 mL were collected from the LSPD fluid before administration as well as from the dialysate as

described above in the PK Section. Within 1 h after collection, samples were stored frozen at −18 °C or below pending analysis.

2.2.6.2. Ammonia analysis. The concentration of ammonia in collected plasma, LSPD and dialysate samples was measured by an enzymatic method using Cobas 6000 analyzer (Hoffmann-La Roche, Basel, Switzerland) with the ammonia kit from Hoffmann-La Roche (catalogue number 20766682322). The measurement of ammonia in plasma was as described in the kit instructions. The method was adapted and qualified for ammonia analysis in medium and high dose LSPD.

The measurement of ammonia in plasma samples was performed within 2 h from blood collection for most of the samples. The results obtained for the few samples analyzed outside this time frame, did not deviate from the other results.

The LSPD and dialysate samples were pretreated as follows before measurements on the Cobas 6000 analyzer. The solutions were added 15% Triton X100 at a 2:1 volume ratio yielding a Triton X100 concentration of 5%. The samples were then incubated at room temperature for 1 h and placed in an ultrasonic bath for 10 min. A series of dilutions of ammonia in phosphate-buffered saline were prepared and the measured mean concentration of ammonia were used to calibrate the ammonia concentration in LSPD (medium and high doses) and in the dialysate samples.

2.2.6.3. Calculations. Individual plasma and dialysate ammonia concentration *vs.* time profiles were subjected to non-compartmental analysis similar to the PK analysis. When the plasma, LSPD or dialysate concentration of ammonia was below the LLOD (6.4 µM for plasma, 36.6 µM for medium dose LSPD and 40.8 µM for high dose LSPD), the data point was entered as zero for the calculations. C_{max} , T_{max} and AUC_{0-3} were estimated as explained under the PK section.

Ammonia clearance in the peritoneal fluid (CL_{NH3}) was calculated as follows (Eq. (7)):

$$CL_{NH3} = C_{NH3, dial, 3h} \times Volume\ LSPD\ recovered / plasma\ ammonia\ AUC_{0-3} \quad (7)$$

Where $C_{NH3, dial, 3h}$ is the ammonia concentration in the dialysate measured at 3 h.

2.2.7. Toxicological testing

2.2.7.1. Clinical signs. All visible signs of ill health and any behavioral changes of the Göttingen minipigs were recorded daily prior to dosing and during the study conduct. In addition, a dose related observation was performed 1 h (± 15 min) after end of dosing. Any deviation from normal was recorded. It was also recorded if any Göttingen minipig showed mortality/morbidity at the start and end of the day.

2.2.7.2. Body weight. Starting on arrival, the animals were weighed twice weekly during the pre-treatment period. During the dosing and recovery period, the animals were weighed daily in the morning under fasted conditions and this value was used for the calculation of the daily dose. Moreover, the animals were weighed at necropsy.

2.2.7.3. Electrocardiography (ECG). Before start of treatment and on a single day during the treatment period (day 5), the animals were subjected to electrocardiographic examination using Marquette MAC 3500 (GE Healthcare, Chicago, IL). Furthermore, the recovery animals were re-examined on a single day during the recovery period (days 22/23). The electrocardiographic recordings were performed approximately 1 and 6 h (± 30 min) after end of dosing. Before start of treatment and during the recovery period, recordings were performed at approximately the same time of days as those done during the dosing period. The electrocardiograms were recorded with the animal placed in a sling. A 6-lead system (axial leads) consisting of the bipolar leads I, II and III, and of the augmented leads aVL, aVR and

aVF was used. The speed of the paper was 50 mm/s and amplification 1 mV/cm. The minimum recording period was approximately 15 s.

The P-wave duration, PR, QRS, QT, and RR intervals were measured from preferably lead II. The result for PR, QRS and QT were the average of the measurements of three subsequent beats, whereas the result for RR was the average of ten subsequent beats. The heart rate (beats per min) was calculated on basis of the recordings. To compare QT measurements at different heart rates, a rate correction of the QT interval was calculated using the Fridericia method (QTcF) [19].

2.2.7.4. Ophthalmoscopy. Before start of treatment and on a single day during the treatment period (day 9), ophthalmoscopy was performed on all animals. Furthermore, the recovery animals were reexamined on a single day during the recovery period (days 22/23). After application of tropicamide 1% solution (Mydracil, Alcon, Geneva, Switzerland), both eyes were examined with an indirect ophthalmoscope and a portable slit-lamp microscope.

2.2.7.5. Clinical pathology. Before start of treatment and on a single day during the treatment period (day 9), blood samples were taken from all animals. Furthermore, blood samples were taken from all recovery animals on a single day during the recovery period (days 23/24). The animals were fasted overnight before blood samples were taken but water was available. Blood samples were drawn from the jugular vein/bijugular trunk.

2.2.7.6. Hematology and coagulation. For hematology, at least 2.5 mL K₃EDTA -stabilized blood was taken.

For the coagulation tests, 1.8 mL citrate stabilized blood was taken. The following parameters were investigated: hemoglobin, red blood cell count, reticulocyte count, hematocrit, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, white blood cell count, differential leucocyte count (neutrophils, lymphocytes, eosinophils, basophils, monocytes), platelet count (ABX Pentra DX120SPS, Horiba, Kyoto, Japan), activated partial thromboplastin time with synthetic phospholipids, prothrombin time, fibrinogen (IL Test™ / ACL Top™, Instrumentation Laboratory, Bedford, MA).

2.2.7.7. Clinical chemistry. Approximately 3 mL of blood was taken for clinical chemistry in plain glass tubes for serum. The following parameters were investigated: alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, bilirubin (total), gamma-glutamyl transferase, cholesterol, triglycerides, carbamide (urea), creatinine, glucose, sodium, potassium, calcium, magnesium, inorganic phosphorous, chloride, protein (total), albumin (Cobas 6000).

2.2.7.8. Urinalysis. Before start of treatment, urine was collected from all animals. At termination of treatment, urine was collected from all animals (except for the recovery animals) in connection with the necropsy. Furthermore, urine samples were taken from all recovery animals in connection with the necropsy. During urine sampling (before start of treatment), the animals were housed individually in metal cages with a floor area of at least 0.72 m². Urine samples were collected overnight from the stainless-steel tray under the cage. No afternoon meal was given, and only water was available during the sampling period. The volumes of the urine samples were recorded and aliquots of up to 10 mL were saved for the analyses.

The following urine parameters were investigated: volume, color (visual inspection), specific gravity, pH, protein, leucocytes, nitrite, blood, glucose, ketones, bilirubin, urobilinogen (Siemens Multistix 10 SG, Clinitek Advantus Analyzer, Siemens Healthineers, Erlangen, Germany).

2.2.7.9. Necropsy. At necropsy, macroscopic examination was performed by observing the appearance of the organs and tissues *in situ*. Any macroscopic change was recorded with details of the location,

color, shape and size in Provantis® (version 9.3.0.0, Instem, Stone, UK).

2.2.7.10. Organs and tissues. Either whole organs or selected samples of the indicated organs and tissues were subjected to the procedure below. Weights were recorded in Provantis®.

Paired organs were weighed together. The relative organ weights (*i.e.*, as a percentage of the body weight and as a percentage of the brain weight) were calculated for each animal.

All tissues were initially fixed in phosphate buffered neutral 4% formaldehyde except for the eyes and testes (Modified Davidson's fixative). The lungs were infused with fixative at necropsy.

2.2.7.11. Microscopic examination. Samples from all animals were trimmed and representative specimens were taken for histological processing. The specimens were embedded in paraffin and cut at a nominal thickness of approximately 5 µm, stained with hematoxylin and eosin, and examined under a light microscope. All organs and tissues were examined microscopically. Histological alterations were graded on a 5-level scale (minimal, mild, moderate, marked, and severe).

2.2.8. Ammonia infusion study

In this part of the study, the safety and efficacy of LSPD was investigated in an artificially induced hyperammonemic pig model. Domestic pigs (9–14 kg, *n* = 2 per group) were anesthetized (sevo-flurane inhalation), endotracheally intubated, kept warm (37.5 °C – 38.5 °C), and thoroughly monitored throughout the study (ECG, in- and expired gas composition, intra-arterial blood pressure, heart and respiratory rates, eye reflexes). Buprenorphine (0.02 mg/kg intravenous) and meloxicam (0.4 mg/kg intramuscular) were administered for pre-emptive analgesia. A pediatric PD multi-perforated catheter (8.5 French, Cook Medical, Bloomington, IN) was inserted in the lower abdominal quadrant of the anesthetized pigs by minimal sterile surgery. After surgery, the pigs were continuously infused for 4 h with an ammonium chloride solution at 1 mmol/kg/h (11.5 mL/kg/h) and their ammonium plasmatic levels were monitored (PocketChem BA PA-4140, Arkray, Japan) at allotted time points (baseline and at *t* = 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3.5, 4 h). Pre-warmed (37 °C) LSPD or Physioneal® 35 1.36% w/v were temporarily injected (40 mL/kg equivalent to 192 and 234 mg/kg citric acid and lipids, respectively) in the peritoneal cavity for 3.5-h dwell periods. At the end of the experiment, the dialysate was drained out of the peritoneal cavity by gravity and soft suction. Subsequently, the pigs were anesthetized and euthanized by intravenous administration of pentobarbital (80 mg/kg).

2.3. Statistics

The statistical calculations were carried out by SigmaPlot (version 13.0) and Microsoft Excel 2016 (linear regression analysis). Three or more groups were compared using one-way ANOVA and Tukey's post-hoc test. Two groups were compared using Student's *t*-test. A *p*-value of < 0.05 was deemed statistically significant.

2.4. Safety margin of FIH dose and anticipated citric acid exposure in patients

Due to the limited exposure to lipids, the safety margin of the LSPD dose selected for the FIH study was estimated for citric acid using a PK-guided approach. The first step consisted in calculating the human equivalent dose (HED) as follows (Eq. (8)):

$$\text{HED} = (\text{CL}_{\text{patients}}/\text{F}) \times \text{AUC}_{\text{inf,day10,NOAEL}} \quad (8)$$

Where CL_{patients} represents the clearance of citric acid in cirrhotic and non-cirrhotic patients already published in the literature (*i.e.*, 265 and 592 mL/h/kg, respectively) [20]; F is the bioavailability assumed to be 1 (most conservative estimate giving the highest exposure);

$AUC_{inf,day10,NOAEL}$ is the area under the curve from 0 to infinity at day 10 following the No Observed Adverse Effect Level (NOAEL) as defined in this repeated dose toxicity study. The calculation of AUC_{inf} is described under the PK section.

In a second step, the safety margin of the starting dose of LSPD in humans was calculated as follows (Eq. (9)):

$$\text{Safety margin} = \text{HED}/\text{FIH LSPD starting dose} \quad (9)$$

Finally, the exposure to citric acid anticipated in patients in the FIH trial was estimated using the starting dose of LSPD and $CL_{patients}$ (obtained from the literature) according to Eq. (8) [20].

3. Results and discussion

3.1. Pharmacokinetics

3.1.1. Pharmacokinetics in plasma

During the extemporaneous preparation of LSPD, liposomes are loaded with citric acid using an osmotic shock with an encapsulation efficiency of ~12% [13]; substantial amounts of deprotonated citrate remain in the external phase and contribute to the osmolarity of the formulation. As shown in our previous work, encapsulated citric acid is the fuel for ammonia capture capacity of LSPD fluids. Moreover, Braide et al., reported that citrate supplementation of a PD fluid improves ultrafiltration and clearance of small solutes in single dwells although the exact mechanism of action remains unknown [21]. Consequently, the PK of citric acid following LSPD treatment was assessed. Upon single and multiple administrations of the medium and high dose LSPD fluids, maximum citric acid plasma concentrations were reached approximately 1 h post-dose (Fig. 2A, Supplementary Fig. S1A, Table 2). The high dose led to higher plasmatic levels than the medium dose both on days 1 and 10, most likely due to the absorption of unencapsulated citric acid from the peritoneum. Mean plasma concentrations subsequently declined in a biphasic manner and returned to pre-dose levels between 7 and 12 h post-dose similarly for both doses and treatment days. The extent of exposure to citric acid increased in a dose-dependent manner as the C_{max} increased proportionally with the dose on days 1 and 10 ($p < 0.01$); the increase in AUC_{0-24} reached significance on day 1 and showed a trend towards significance on day 10 ($p = 0.061$, Supplementary Table S1). For males and females combined, the mean C_{max} was approximately 109 vs. 77.0 $\mu\text{g/mL}$ and the mean AUC_{0-24} ranged between 654 and 706 vs. 473–570 $\mu\text{g}\cdot\text{h/mL}$ for high vs. medium dose LSPD (Table 2). The mean half-life ($t_{1/2}$) of citric acid was 2–3 h, and similarly to CL/F and V_z/F (approximately 200 mL/h/kg and 700–800 mL/kg , respectively), it was not significantly different between the two doses on the same treatment day (Supplementary Table S1). The plasma C_{max} and AUC_{0-24} were considerably lower than the reported safe maximum values in critically ill cirrhotic patients (307 $\mu\text{g/mL}$ and 903 $\mu\text{g}\cdot\text{h/mL}$ [20]). Importantly, there was no systemic accumulation of citric acid in minipigs following daily intraperitoneal infusions of medium and high dose LSPD fluids over 10 days (Supplementary Table S2). The plasma PK profile of citric acid was comparable in males and females as no significant gender differences in the plasma concentration curves were observed within the same dose (Supplementary Fig. S2, Supplementary Table S3). Since the risk to suffer from HE affects both genders equally [22], the absence of a gender effect based on individual PK data provides assurance that both female and male patients meeting the inclusion criteria could be safely included in the first clinical trial.

The linear PK of citric acid following intraperitoneal administration of LSPD is an important property to accurately select the starting dose in patients. This is especially relevant since the European Medicine Agency recently revised its guideline on FIH trials [23]. While the previous version of the guideline focused on the emergence of non-clinical adverse events for estimating safe doses in FIH studies, the current revision advocates to consider the profile of the dose/exposure-

response curves within the anticipated therapeutic range (e.g., steepness, plateau of the curve) and a clear limit to exposure must be provided in the protocol.

Intraperitoneally applied liposomes enter the lymphatics at the diaphragm and are drained into the circulatory system at the jugular-subclavian junction [24]. After a single administration of LSPD in minipigs, the systemic absorption and elimination of DPPC were slow; DPPC plasma concentrations remained above baseline levels at the end of the dosing interval (Fig. 2B, Supplementary Fig. S1B). Therefore, the terminal elimination phase of DPPC could not be adequately characterized on day 1 and all λ_z -related PK parameters (i.e., AUC_{inf} , CL/F and V_z/F) should be interpreted with caution (Table 3). On day 10, DPPC plasma concentrations declined in a biphasic manner and returned to pre-dose levels after 16 to 24 h post-dose in both treatment groups. The rate and extent of exposure to DPPC in minipigs as well as systemic elimination were comparable across doses as no significant differences in C_{max} , AUC_{0-24} , t_{max} , $t_{1/2}$, CL/F , or V_z/F were observed between the two doses on the same treatment day (Supplementary Table S4). The mean C_{max} ranged between 112 and 130 $\mu\text{g/mL}$ on day 1 and 72.8–105 $\mu\text{g/mL}$ on day 10. The mean AUC_{0-24} ranged between 1905 and 1959 $\mu\text{g}\cdot\text{h/mL}$ and 798–1136 $\mu\text{g}\cdot\text{h/mL}$ on day 1 and day 10, respectively (Table 3). On day 10, the mean half-life in both groups was between 11.3 and 15.4 h. The DPPC plasma concentration curves were not statistically different within a given dose between male and female animals (Supplementary Fig. S3, Supplementary Table S5). Furthermore, following multiple intraperitoneal administrations of medium and high dose LSPD *q.d.*, there was no systemic accumulation of DPPC in minipigs (Supplementary Table S2).

Interestingly, the overall extent of exposure appeared to decrease on day 10 compared to day 1; mean C_{max} values were comparable (Table 3). The difference in the DPPC AUC_{0-24} between days 1 and 10 was only significant in the medium dose group (Supplementary Table S4). This trend towards lower systemic exposure following multiple dosing might be related to the accelerated blood clearance phenomenon. The latter has been reported for PEGylated liposomes upon repeated administrations in several species (rats, rhesus monkeys, mice, minipigs, dogs) and is likely related to the opsonization of these vesicles by anti-PEG immunoglobulin M antibodies [25–29]. In general, the extent of exposure to DPPC inversely correlated with the amount of DPPC recovered at the end of intraperitoneal treatments with LSPD (Fig. 3A), which in turn depended on the volume of fluid drained from the abdominal cavity (Fig. 3B).

To estimate the maximum recoverable DPPC dose, the recovered DPPC was compared to the recovered volume of LSPD fluid (Fig. 3B). Based on a linear regression, it was estimated that draining out 100% of LSPD would remove approximately 77% of the total lipids administered, implying that about 23% of the dose is absorbed during the dwell time of 3 h. Analogous calculations for cholesterol yielded a similarly low absorption for cholesterol (approx. 21%) assuming full LSPD removal (Supplementary Fig. S4). Therefore, most liposomes remain confined in the peritoneal space during the dwell time. In contrast, draining the entire LSPD volume would theoretically remove about 33% of citric acid (Supplementary Fig. S5), indicating that about two thirds the citric acid dose was absorbed in the systemic circulation and subsequently eliminated. We hypothesize that in the clinical practice a shorter dwell time (e.g., 2 h) and an efficient drainage of LSPD via the introduction of a washing step may significantly reduce the overall exposure to liposomes in the systemic circulation.

The rate (C_{max}) and extent of exposure (AUC) to cholesterol in minipigs were similar following repeated intraperitoneal infusions of medium and high dose LSPD (Supplementary Table S6, no statistically significant differences). In fact, cholesterol concentrations following both doses remained around baseline levels and λ_z -related PK parameters could not be calculated. The apparent differences in observed absorption between cholesterol and DPPC could be related to the lower amount of cholesterol in the formulation and higher endogenous levels.

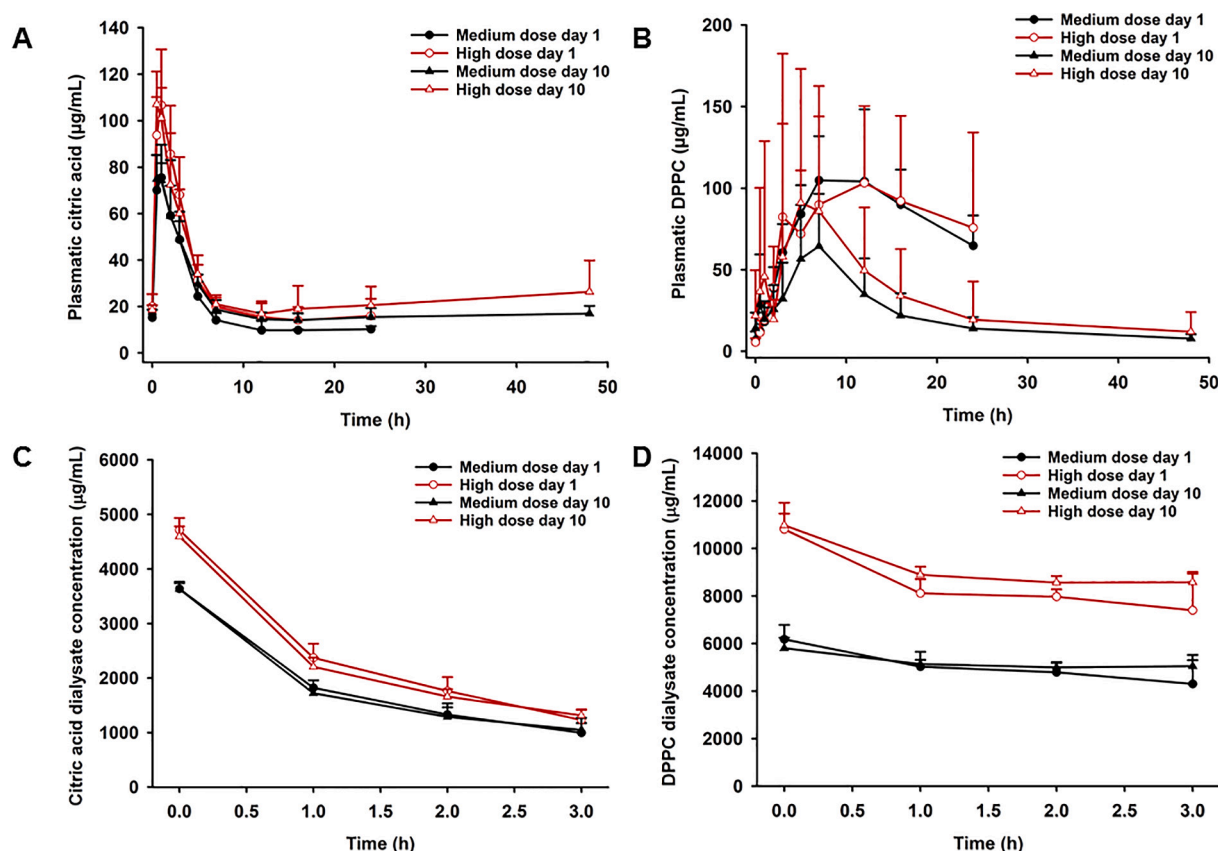


Fig. 2. Mean PK profile of citric acid and DPPC in plasma and dialysate of LSPD-treated minipigs following single (day 1) and multiple doses (day 10). Plasma citric acid concentrations over time after LSPD administration (A). Plasma DPPC concentrations over time after LSPD administration (B). Peritoneal citric acid concentrations over time after LSPD administration (C). Peritoneal DPPC concentrations over time after LSPD administration (D). On both treatment days, dialysate concentrations were significantly higher for high dose LSPD than medium dose for citric acid up to 2 h post-dose ($p < 0.01$, C) and for DPPC for all investigated time points ($p < 0.001$, D). Mean + SD ($n = 7-8$).

Following multiple intraperitoneal infusions of medium and high doses LSPD daily over 10 days, there was no plasma accumulation of cholesterol (Supplementary Table S2).

3.1.2. Pharmacokinetics in dialysate

Significant differences between medium and high dose LSPD were observed for C_{max} and AUC_{0-3} of dialysate citric acid and DPPC (Fig. 2C and D, Table 4, Supplementary Tables S7 and S8). Citric acid concentrations in the peritoneal fluid declined more rapidly than DPPC indicating the more rapid absorption in the systemic circulation. Indeed on days 1 and 10, only 7% to 37% of the dose of citric acid was recovered in the dialysate at the end of the PD session whereas the percentage of recovered DPPC in the dialysate varied between 21% and 96% and correlated well with the volume of dialysate recovered at the end of the PD session (Supplementary Table S9). The low dialysate recovery and hence the higher exposure to lipids achieved in certain cases was likely related to the omentum wrapping of the catheter or to the localization of the catheter inside the peritoneal space, which probably impeded optimal drainage. In patients, however, the catheter

would not be implanted surgically. A catheter for paracentesis would be used and it is expected that the recovery of the dialysate would improve and be less variable. The PK profile of citric acid and DPPC in the peritoneal space was linear both on days 1 and 10 at the medium and high LSPD doses. There seemed to be no accumulation in the peritoneal cavity on day 10 (Supplementary Table S2). Furthermore, the dialysate citric acid and DPPC concentrations of male and female minipigs were not significantly different (Supplementary Figs. S6 and S7).

3.2. Efficacy

Following intraperitoneal administration, ammonia C_{max} and AUC_{0-3} were significantly higher in the dialysate of minipigs in the medium and high dose LSPD groups than the control group on both treatment days (Table 5, Supplementary Table S10). In addition, these two PK parameters dialysate ammonia curves were significantly higher for the high dose than the medium dose (Fig. 4A and B). Therefore, there was a citric acid dose-dependent increase in ammonia capture between the two groups of LSPD-treated minipigs. This indicates that

Table 2

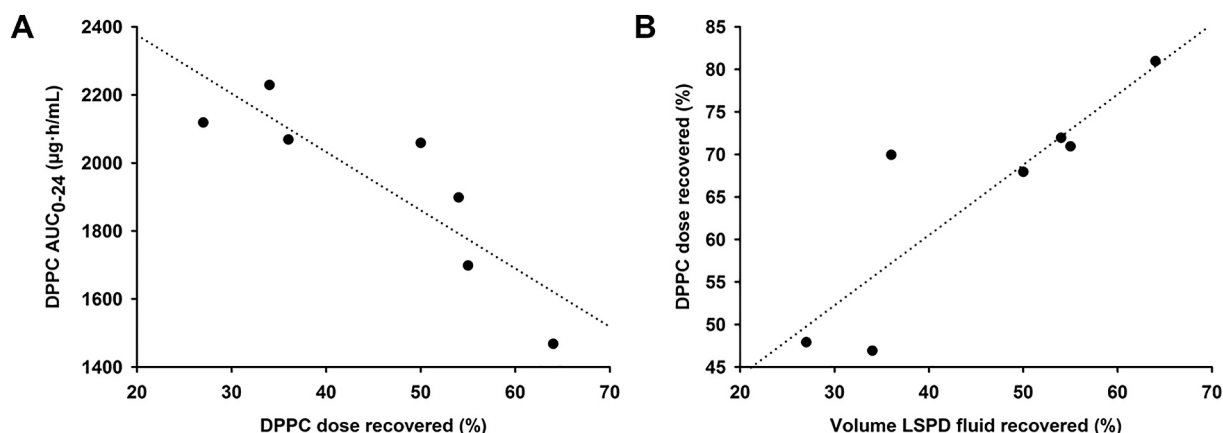
Mean PK parameters of plasma citric acid ($n = 7-8$). Statistically significant differences are reported in Supplementary Table S1.

Dose	Day	C_{max} (µg/mL)	t_{max} (h)	$t_{1/2}$ (h)	AUC_{0-3} (µg·h/mL)	AUC_{0-24} (µg·h/mL)	AUC_{inf}^a (µg·h/mL)	AUC_{ext} (%)	CL/F^1 (mL/h/kg)	V_z/F^1 (mL/kg)
Medium	1	76.6	0.786	2.32	179	473	508	7.07	232	785
Medium	10	77.1	0.786	2.98	182	570	639	10.5	185	787
High	1	110	0.875	2.04	251	654	697	6.61	226	683
High	10	108	0.625	2.58	235	706	782	9.52	200	753

^a Based on AUC_{0-24} .

Table 3Mean PK parameters of plasma DPPC ($n = 4-8$). Statistically significant differences are reported in Supplementary Table S4.

Dose	Day	C_{max} ($\mu\text{g/mL}$)	t_{max} (h)	$t_{1/2}$ (h)	AUC_{0-3} ($\mu\text{g}\cdot\text{h/mL}$)	AUC_{0-24} ($\mu\text{g}\cdot\text{h/mL}$)	AUC_{inf} ($\mu\text{g}\cdot\text{h/mL}$)	AUC_{ext} (%)	CL/F (mL/h/kg)	V_z/F (mL/kg)
Medium	1	112	9.28	16.7	91.5	1905	3375	42.9	59.6	1410
Medium	10	72.8	4.67	15.4	76.2	798	1530	12.4	135	2910
High	1	130	11.6	15.8	103	1959	2755	44.5	142	3110
High	10	105	7.38	11.3	108	1136	1405	10.3	304	3994

**Fig. 3.** DPPC exposure vs. LSPD recovery in minipigs. DPPC AUC_{0-24} in plasma vs. recovered DPPC doses ($R^2 = 0.7478$, A). Relationship between recovered amount of DPPC (expressed as % of the dose administered) and recovered volume of LSPD ($R^2 = 0.7523$, B). Data presented from medium dose LSPD on day 1.

the extraction of ammonia in the dialysate could be well controlled by the citric acid dose in LSPD fluids. A similar dose effect of LSPD fluid citric acid on dialysate ammonia concentration was observed in healthy rats [13]. However, in contrast to healthy rats, where a linear increase in ammonia uptake was seen up to 4 h [13], the ammonia sequestration leveled off after 1 h in minipigs. Importantly, dialysate ammonia C_{max} and AUC_{0-3} were not significantly different on the first and tenth day of daily LSPD infusions. Furthermore, there were no gender differences in the DPPC dialysate ammonia concentration curves within the same dose and treatment day.

No changes in plasma ammonia concentrations were observed upon LSPD administration (Fig. 4C). This result was expected as in healthy animals ammonia levels are low due to an efficient hepatic clearance [30,31]. We previously reported in bile duct ligated rats (a liver disease model) that LSPD was indeed efficient to reduce hyperammonemia and associated HE [13]. Similarly, a recent study found that an ammonia-sequestering treatment efficiently decreased systemic ammonia levels in hyperammonemic rats but failed to show effects in healthy control rats, potentially due to difficulties in reliably quantifying low ammonia concentrations and/or because of acid-base homeostasis-regulating ammoniagenic pathways activated in response to hypoammonemia [16,32–35]. To confirm that LSPD would remove excessive plasma ammonia in a large animal model, we conducted a pilot experiment in which ammonia was continuously infused in domestic pigs to reach a hyperammonemic state of approximately 200 μM . In this setup, we showed that LSPD enhanced the elimination of ammonia, reducing the

plasmatic concentrations by 30% within 3 h from infusion onset, compared to a 10% increase observed in the control group treated with Physioneal® 35 1.36% w/v (Supplementary Fig. S8). These preliminary outcomes support the initial proof of concept in rodents and encourage the future clinical development of LSPD as a first-line support for acute and life-threatening hyperammonemia in urea cycle defects and related liver deficiencies. In pediatric patients, the treatment of acute hyperammonemia is currently challenging because of the delayed onset of action of metabolic treatments and the invasiveness of hemodialysis [1,3]. Therefore, LSPD as a rapidly acting and mildly invasive treatment modality promises to improve the overall prognosis of these patients. Mean ammonia peritoneal fluid clearance (CL_{NH_3}) measured in minipigs ranged between 5.33 and 11.5 mL/min (Supplementary Table 11) following single and multiple doses LSPD. This result was in the same

Table 5PK parameters of peritoneal fluid ammonia (mean \pm SD; $n = 6-8$). Statistically significant differences are reported in Supplementary Table S10.

Group	C_{max} (μM)		AUC_{0-3} ($\mu\text{mol}\cdot\text{h/mL}$)	
	Day 1	Day 10	Day 1	Day 10
Control	67.5 \pm 13.9	57.5 \pm 12.5	15.6 \pm 3.41	13.0 \pm 1.71
Medium dose	251 \pm 65.3	199 \pm 49.0	53.1 \pm 13.8	45.3 \pm 12.0
High dose	344 \pm 40.6	323 \pm 76.2	79.0 \pm 11.8	79.5 \pm 19.5

Table 4Mean PK parameters of dialysate citric acid and DPPC ($n = 7-8$). Statistically significant differences are reported in Supplementary Tables S7 and S8.

Dose	Day	Citric acid		DPPC	
		C_{max} ($\mu\text{g/mL}$)	AUC_{0-3} ($\mu\text{g}\cdot\text{h/mL}$)	C_{max} ($\mu\text{g/mL}$)	AUC_{0-3} ($\mu\text{g}\cdot\text{h/mL}$)
Medium	1	3641	5372	6197	15,070
Medium	10	3636	5244	5880	15,611
High	1	4715	6963	10,813	25,128
High	10	4598	6679	10,988	27,230

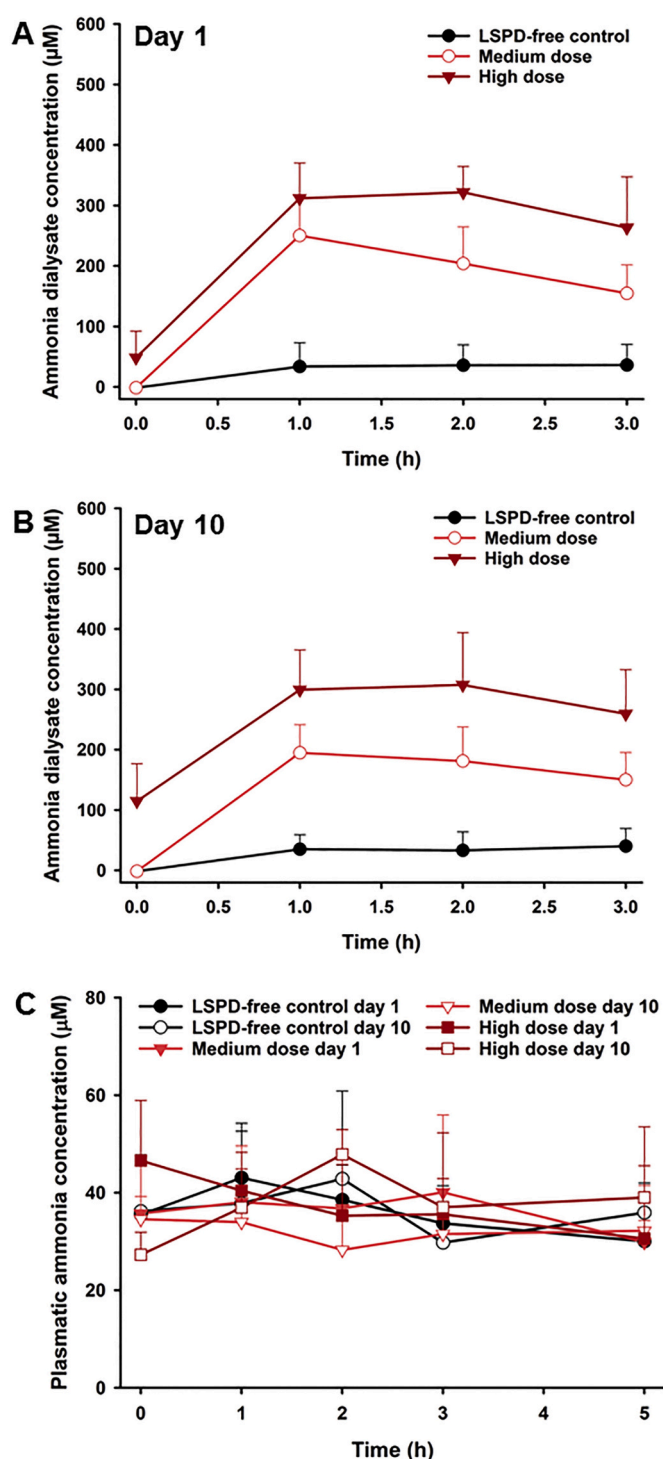


Fig. 4. *In vivo* ammonia extraction by LSPD in minipigs. Ammonia concentrations in dialysate fluid containing ascending doses of citric acid during 3 h of PD on days 1 (A) and 10 (B). Plasma ammonia concentrations during and after 3 h of PD on days 1 and 10 (C). Dialysate ammonia concentrations were significantly higher for medium and high dose LSPD than control PD starting from 1 h post-dose ($p < 0.01$, A and B). No statistically significant differences in plasma ammonia were observed for LSPD over control PD (C). Mean + SD ($n = 6-8$).

range compared to other dialysis modalities used as standard of care in pediatric patients with acute hyperammonemia for which ammonia clearance varied between 2.5 and 21.5 mL/min [36]. It is therefore anticipated that the performance of LSPD in clearing ammonia can at

least reach that of hemodialysis. This is yet to be confirmed in future clinical trials.

3.3. Toxicology

The toxicity of LSPD was assessed on minipigs mainly because anaphylactoid-like reactions, a serious adverse event for colloidal formulations, are not reported in rodents [37] and due to the complexity of performing repeated peritoneal administrations in small size animals. The parameters evaluated in the study included clinical signs, body weight, food consumption, ophthalmoscopy, clinical pathology (blood and urine) and electrocardiography. At necropsy, a full pathological evaluation including organ weight and histopathological examination of selected tissues was performed.

The medium and high LSPD doses were well-tolerated in both males and females. No LSPD-related changes in food consumption, clinical pathology, and organ weights were found. All animals had a normal body weight gain throughout the study except for the male medium dose group, which had a significantly lower body weight gain from Day 1 to Day 12 (Supplementary Tables S12 and S13). This finding was judged of no toxicological importance as it was only observed in males from a single treatment group. Furthermore, there were no changes in coagulation parameters and most biochemical and blood parameters (Supplementary Tables S14-S17). The lack of effect of citrate on blood coagulation and clinical chemistry parameters is an important finding as the latter is known to chelate ionized calcium and may be associated to bleeding, hypocalcemia and metabolic acidosis, which would represent adverse effects of special interest for LSPD [38].

Treatment with LSPD had no important influence on the electrocardiography (Supplementary Tables S18 and S19). A significant decrease in heart rate and a significant increase in the RR interval was observed in females on day 5 at 1 h post-high dose LSPD. At 6 h post-dose, a significant increase in QT was observed in the same group but the corrected QTcF showed no significant changes. These observations were judged of no toxicological importance as no clear trend was observed across the two timepoints. Overall, based on cardiovascular parameters, white blood cells and body temperature, no anaphylactoid-like reactions were reported in minipigs treated with LSPD in accordance with our previous study [13].

At macroscopic examination, adhesions extending from the catheter/implantation site to various tissues and organs were recorded in one or more animals in all investigated groups. Other macroscopic findings most likely related to catheter implantation were a grey-white focus on the spleen in one animal, pink discoloration of the peritoneum and red discoloration of the serosa of the cecum in single or a few animals. The tissue of the thymus of several animals treated with the liposomal formulation were found to be firm to the touch.

The histopathologic analysis of the spleen, liver, and kidney did not show treatment-related toxicity (Supplementary Table S20). Microscopic findings considered to be related to the liposomal formulation included minimal to strong presence of vacuolated macrophages (mainly microvesicular vacuolation) in lymph nodes of the LSPD-treated animals. Furthermore, a minimal to mild infiltration of similar vacuolated macrophages was seen in various tissues of some LSPD-treated animals irrespective of the dose. Vacuolated macrophages were only seen in a few tissues of one recovery animal (male) receiving LSPD high dose, indicating on-going recovery. These findings were expected as macrophages are involved in the clearance of liposomes [39–41]. A recent study identified that intraperitoneally applied liposomes mainly entered the lymphatics at the diaphragm and were drained via the thoracic lymph to the jugular-subclavian junction, accumulating preferentially in mediastinal lymph nodes [24]. This report is in accordance with our finding that the firm lymph nodes, which were subsequently sampled and showed vacuolated macrophages, were adjacent to the thymic tissue, while no histopathologic findings were made in the mesenteric lymph nodes of the treated animals

Table 6
HED – PK-guided approach (citric acid).

PK parameters in minipigs		
AUC _{inf,day10,NOAEL} (μg·h/mL)		782
PK parameters in patients	Cirrhotic patients	Non-cirrhotic patients
CL _{patients} (mL/h/kg)	265 ^a	592 ^a
Estimated HED & starting dose	Based on CL _{cirrhotic patients}	Based on CL _{non-cirrhotic patients}
HED (mg/kg)	207	463
LSPD starting dose (mg/kg)	53.1	53.1
Safety Margin	x 3.9	x 8.7

HED = (CL_{patients} / F) × AUC_{inf,day10,NOAEL} with F = 1.

Safety margin = HED / LSPD starting dose.

^a Data taken from reference [20].

(Supplementary Table S20). Furthermore, the aforementioned study reported that liposomes with a high degree of PEGylation (6 mol% of mPEG-DSPE) are less prone to accumulate in lymph nodes compared with non-PEGylated liposomes.

Microscopic findings considered to be related to the catheter implantation procedure and peritoneal administration of the liposomal or the control formulations included an inflammatory response around the catheter implantation sites, in the omentum and peritoneal samples, and in the peritoneal/serosal surfaces of several abdominal organs (e.g., liver, pancreas, spleen) as well as in additional tissues (e.g., muscle tissue) adjacent to collected tissues and organs. Other microscopic findings observed in the study animals were considered incidental and/or within the range of expected findings in Göttingen minipigs of this age.

3.4. Rationale for dose selection and anticipated exposure to citric acid/lipids in humans

For all groups of minipigs, the 10-day duration of the repeated dose toxicity covered more than the maximum time during which clinical exposure is anticipated in liver decompensated patients. Overall, both formulations were safe. High dose LSPD was defined as the NOAEL even though a maximum tolerated dose has not been reached. Nevertheless, considering the lack of supportive data on the permeability of the peritoneal membrane in liver impaired patients, and the possibility of liposomes' associated CARPA reactions, the medium dose LSPD (3.54 mg/mL citric acid / 6.49 mg/mL lipids) given at 15 mL/kg was selected as a starting dose in patients, which represents a total dose of 53.1 mg/kg citric acid (Table 6) which is considerably lower than the safe citric acid dose in critically ill patients (192 mg/kg [20]). Based on a PK-guided approach, a corresponding HED of 207–463 mg/kg was calculated according to citric acid clearance in cirrhotic and non-cirrhotic patients [20]. Considering that the safety profile of citric acid is already well known, the resulting safety margin above 3, gives enough confidence that the first dose of LSPD should not trigger serious adverse reactions in patients. Even though no effects on hematology or clinical chemistry were found during the toxicity program, these cannot be excluded in a clinical setting. Indeed, citrate is a well-known anticoagulant; a close monitoring of the coagulation parameters, ionized calcium levels, and acid-base status (blood gas analysis panel) is thus recommended to mitigate the risks associated to citrate during the FIH trial with LSPD, especially in liver impaired patients.

The extent of exposure to citric acid (AUC_{inf}) following intraperitoneal administration of LSPD at the starting dose, is expected to range between 89.7 and 200 μg·h/mL. This is lower than the value estimated from the literature data in critically ill cirrhotic patients (1008 μg·h/mL [20]) and minipigs at the NOAEL (782 μg·h/mL). The risks of citric acid related adverse events are therefore expected to be low also considering that citrate is the anticoagulant of choice used in patients undergoing extracorporeal dialysis [42,43]. Once LSPD's safety

is confirmed in patients and preliminary PK and efficacy results are gathered, an appropriate dosing regimen (including dose level, length of dwell time, number of LSPD sessions per day) will be established prior to the phase II clinical proof of concept study.

4. Conclusions

Based on non-clinical proof of concept studies in bile duct ligated rats and in artificially induced-hyperammonemic domestic pigs, LSPD is a promising investigational treatment modality for hyperammonemia-associated disorders leading to brain edema or hepatic encephalopathy (e.g., metabolic disorders affecting the urea cycle, liver injuries). In this GLP study, we performed an in-depth analysis of the PK, ammonia uptake, and toxicological properties of peritoneally administered transmembrane pH-gradient liposomes in Göttingen minipigs. Once daily sessions with LSPD for ten days resulted in favorable PK with rapid absorption and elimination of citric acid and a low systemic exposure to DPPC. Transmembrane pH-gradient liposomes concentrated ammonia in the peritoneal space to a higher extent than a liposome-free PD fluid. The study further confirmed the safety of LSPD in minipigs, one of the most sensitive species to colloidal formulations. The results of this work indicate a positive pharmacokinetic and toxicological profile for LSPD and support a clinical investigation in hyperammonemic patients.

Declaration of Competing Interest

JCL is a cofounder and shareholder of Versantis AG.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jconrel.2020.08.040>.

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